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(54) Production of 2'-deoxynucleosides and 2'-deoxynucleoside precursors from 2-dehydro-3-deoxy-D-gluconate

(57) This invention relates to a process for preparing 2'-deoxynucleoside compounds or 2'-deoxynucleoside precursors using 2-dehydro-3-deoxy-D-gluconic acid (usually abbreviated as KDG) or its salts as a starting material. A variety of 2'-deoxynucleosides and their analogues are used as a starting material for synthesis or drug formulation in production of an antiviral, anticancer or antisense agent.

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Description

[0001] This invention relates to a process for preparing 2'-deoxynucleoside compounds or 2'-deoxynucleoside precursors using 2-dehydro-3-deoxy-D-gluconic acid (usually abbreviated as KDG) or its salts as a starting material. A variety of 2'-deoxynucleosides and their analogues are used as a starting material for synthesis or drug formulation in production of an antiviral, anticancer or antisense agent.

[0002] Specifically, the invention relates to a method in which KDG or a derivative of KDG is subjected to a decarboxylation step to remove the original carboxy group of KDG. In a preferred embodiment, the KDG used in the method according to the invention is enzymatically produced from D-gluconate or D-glucosaminate.

[0003] 2'-deoxynucleosides and 2'-deoxynucleoside precursors including 2-deoxy-D-ribose are used as starting material for synthesis or drug formulation, for instance, in production of antiviral and anticancer agent. 2'-deoxynucleosides or derivatives thereof and 2'-deoxynucleoside precursors are also used as reagents for research, diagnosis and synthesis of therapeutic antisense molecules.

[0004] In one method of the prior art, deoxynucleosides are generated from biological materials such as testis (WO 99/49074) or yeast or fish sperm by enzymatic cleavage of DNA. This method, however, involves several disadvantages, in particular regarding difficulties of obtaining the starting material in sufficient quantity and quality.

[0005] The main production process of 2-deoxy-D-ribose currently consists in chemical hydrolysis of DNA. In this case, the deoxyribosyl moiety originates in ribonucleotide reductase activity. No synthesis of 2-deoxy-D-ribose from KDG has been yet described.

[0006] In most living cells, deoxyribonucleosides result from a "salvage pathway" of the nucleotide metabolism. The deoxyribose moiety of deoxyribonucleosides is obtained through the reduction of a ribosyl moiety into di- or triphosphate ribonucleotides catalyzed by ribonucleotide reductases. However, the deoxyribose moiety is not recycled, but is degraded into D-glyceraldehyde-3-phosphate and acetaldehyde following the reactions of central metabolism:

- deoxynucleoside is cleaved into deoxyribose-1-phosphate and nucleobase through phosphorolysis mediated by products of the genes encoding thymidine phosphorylase (deoA), purine-nucleoside phosphorylase (deoD), uridine phosphorylase (udp) or xanthosine phosphorylase (xapA).
- deoxyribose-1-phosphate is converted into deoxyribose-5-phosphate through a reaction catalyzed by deoxyribose phosphate mutase (deoB),
- which is further degraded to D-glyceraldehyde-3-phosphate and acetaldehyde through a reaction catalyzed by deoxyribose-5-phosphate aldolase (deoC).

[0007] It has been shown that the deo enzymes also catalyze in vitro the reverse anabolic reactions: Deoxyribose-5-phosphate is obtained in vitro in the presence of purified *Escherichia coli* or *Lactobacillus plantarum* deoxyribose aldolase starting from acetaldehyde and D-glyceraldehyde-3-phosphate (Rosen et al., J. Biol. Chem., 240, (1964), 1517-1524; Pricer, J. Biol. Chem., 235, (1960), 1292-1298). Deoxyribose can also be obtained with acetaldehyde and glyceraldehyde as enzyme substrates, but only with a very low yield (Barbas, J. Am. Chem. Soc. 112 (1990), 2013-2014).

[0008] The patent application WO 01/14566 describes the enzymatic synthesis of deoxynucleosides starting from deoxyribose-1-phosphate through the combined activities of three enzymes of the deo operon, i.e. deoxyribose aldolase, deoxyribomutase and phosphorylase (thymidine or purine nucleoside phosphorylase) in a one-pot reaction, using as starting substrates glyceraldehyde-3-phosphate, acetaldehyde and a nucleobase. D-glyceraldehyde-3-phosphate can be obtained from fructose-1,6-bisphosphate by an enzymatic process.

[0009] The patent application EP 1179598 describes the use of phosphorylase to catalyze the enzymatic production of deoxynucleosides starting from deoxyribose-1-phosphate and nucleobase. The yield of deoxynucleoside synthesis is improved by precipitation of phosphate.

[0010] However, methods using enzymes of the deo operon working in the reverse direction compared to their biological function show low yields, which indicates serious drawbacks for their use.

[0011] In view of the above-described ineffectiveness of the currently applied processes for producing deoxynucleosides and deoxynucleoside precursors, it is an object of the present invention to provide means and methods for the biosynthetic production of deoxynucleosides and deoxynucleoside precursors starting from cheap and commercially available compounds without being dependent on unreliable natural sources.

[0012] In particular, there is a need for alternative methods for the production of deoxynucleosides and deoxynucleoside precursors which allow efficient and economical synthesis of deoxyribonucleosides, by means of which the drawbacks of prior art processes are eliminated.

[0013] The present invention relates to a method for producing 2'-deoxynucleosides and precursors thereof starting from 2-dehydro-3-deoxy-D-gluconic acid (KDG) or its salts and comprising a decarboxylation step.

[0014] In particular, this method is useful for producing 2-deoxy-D-ribose (DRI) as well as synthetically versatile

enamine derivatives of DRI as 2'-deoxynucleoside precursors.

[0015] The decarboxylation step takes place by reacting either KDG or its salts directly, or a derivative of KDG, usually to cleave the C1-C2 bond of the KDG.

[0016] In one embodiment of the invention, KDG or one of its salts undergoes (oxidative) decarboxylation leading to 2-deoxy-D-ribonic acid (DRN) or its salts, itself being further converted into 2-deoxy-D-ribose (DRI) or 2-deoxy-D-ribitol (DRL).

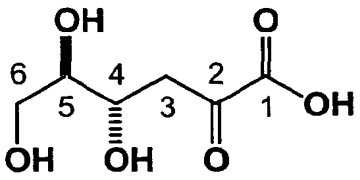
[0017] In another embodiment of the invention, decarboxylation takes place by reacting KDG or its salts with an amine, leading to an enamine derivative. This high energy enamine derivative can be further converted into DRI by hydrolysis.

[0018] In another embodiment of the invention, (oxidative) decarboxylation is carried out on 3-deoxy-D-gluconic acid (DGN) or its salts and/or 3-deoxy-D-mannonic acid (DMN) or its salts as derivatives of KDG, leading to DRI. Production of a mixture of DGN and DMN takes place by reduction of KDG. The decarboxylation is preferably carried out via reaction with hydrogen peroxide.

[0019] In another embodiment of the invention, (oxidative) decarboxylation is carried out on 3-deoxy-D-glucosaminic acid (DGM) or its salts and/or 3-deoxy-D-mannosaminic acid (DMM) or its salts, leading to DRI. Production of a mixture of DGM and DMM takes place from KDG by reductive amination.

[0020] Another aspect of the invention is a convenient and cost-effective method for preparing KDG or its salts to be used in the above methods. This method starts either from D-gluconate or from D-glucosaminate through the use of recombinant enzymes. The invention provides a novel nucleotide sequence encoding a polypeptide having D-gluconate dehydratase activity and a nucleotide sequence encoding a polypeptide having D-glucosaminate deaminase activity.

[0021] The starting material used for the method of the present invention is KDG, represented by formula (I) below or one of its salts, or a protected derivative thereof wherein one ore more of the hydroxyl groups at positions 4, 5 and/or 6 are protected by a protection group known in the art.



(I)

[0022] The term "2'-deoxynucleoside" as used herein relates to 2'-deoxyribonucleosides which are N-glycosides, and wherein the basic N-atom of the nucleobase or nucleobase analog is bound to the anomeric carbon atom of 2-deoxy-D-ribose, or one of its derivatives. Examples of a suitable nucleobase are adenine, cytosine, guanine, thymine, uracil, 2,6-diaminopurine, and hypoxanthine. Examples of nucleobase analogs are 5-azacytosine, 2-chloro-adenine, 5-iodo-cytosine, 8-azaguanine, 5-iodo-uracil, 5-bromo-uracil, 5-fluoro-uracil, 5-ethyl-uracil and 5-trifluoromethyl-uracil.

[0023] The term "2'-deoxynucleoside precursors" as used herein, relates to compounds which can be easily converted into 2'-deoxynucleosides by applying methods known in the prior art. Preferred 2'-deoxynucleoside precursors are 2-deoxy-D-ribose (DRI) or carbohydrate compounds which can be converted into the 2-deoxy-D-ribosyl moiety of 2'-deoxynucleosides, for instance, those established in the prior art 1-phospho-2-deoxy-D-ribose, 5-phospho-2-deoxy-D-ribose and those established by the present invention 2-deoxy-D-ribitol, 2-deoxy-D-ribonic acid, 2-deoxy-D-ribono-1,4-lactone, 1-N-morpholino-3,4,5-trihydroxy-pentene-1, and their derivatives.

[0024] The method of the invention encompasses methods wherein the decarboxylation step is directly carried out on KDG or its salts or on compounds derived from KDG. Preferred KDG derivatives are 3-deoxy-D-gluconic acid, 3-deoxy-D-mannonic acid, 3-deoxy-D-glucosaminic acid and 3-deoxy-D-mannosaminic acid and their respective salts.

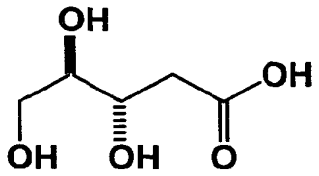
[0025] Furthermore, KDG and its salts or protected forms of these wherein one or more of the hydroxyl groups at the positions 4,5 and /or 6 are replaced by protecting groups known for that purpose in the art are also suitable starting materials for the decarboxylation reaction of the present invention. Unless noted otherwise, any reference to KDG in the following specification embraces protected forms of KDG, just as reference to KDG derivatives is intended to embrace protected forms of these derivatives. Similarly, any reference to the products obtained in the methods of the invention is intended to encompass protected forms of these products. Preferred protection groups for the purpose of the invention are those which replace the respective hydroxyl groups by acetate ester, benzoate ester, allyl ether,

benzyl ether, trityl ether, ter-butyldimethylsilyl (TBDMS) ether, isopropylidene or a benzylidene acetal.

[0026] It should be understood that, depending on suitable reaction conditions for the embodiments of the invention, the carboxylic groups contained in the organic acids used as reactants or obtained as products can be in a protonated form or in their salt form, or may be present in equilibrium. Exemplary salts of these acids are those which have metal or ammonium ions as counterions, particularly alkali metal ions such as sodium and/or potassium.

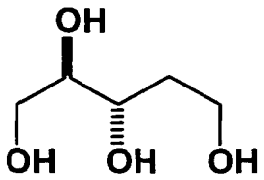
[0027] Most of the carbohydrate compounds and their derivatives described in the present invention exist under several cyclic form but for simplicity reasons have been represented by open chain formulas. It is understood that the present invention encompasses all these isomeric or tautomeric forms.

[0028] In a first embodiment of the invention, KDG or its salts is reacted with hydrogen peroxide and undergoes (oxidative) decarboxylation to 2-deoxy-D-ribonic acid (DRN), a compound of formula (II) or its salts.



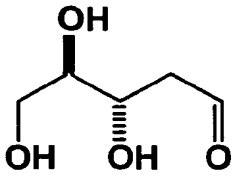
(II)

[0029] The product may be further converted into or 2-deoxy-D-ribitol (DRL), represented by formula (IV)



(IV)

or 2-deoxy-D-ribose (DRI), represented by formula (III)



(III)

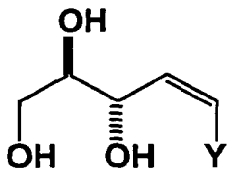
DRN, DRL and particularly DRI are among preferred 2'-deoxynucleoside precursors for the purpose of the present invention. Conversion of DRN to DRI may proceed directly or via DRL as an intermediate.

[0030] Preferably, the preparation of DRN is carried out by oxidative decarboxylation of sodium or potassium 2-dehydro-3-deoxy-D-gluconate in aqueous solution with hydrogen peroxide at room temperature as described in example 5. A general method for the preparation of aldonic acids by oxidative decarboxylation of 2-ketoaldonic acids is described in patent EP 1 038 860 A1.

[0031] Preferably, the preparation of DRL is carried out by hydrogenation of 2-deoxy-D-ribonolactone in aqueous solution with Rhodium catalyst on carbon at a temperature of 130°C under a pressure of 80 bars as described in example 6. 2-Deoxy-D-ribonolactone can be easily prepared by converting a 2-deoxy-D-ribonate (DRN salt) into 2-deoxy-D-ribonic acid, which is in equilibrium with its lactonic form in aqueous solutions (Han, Tetrahedron. 1993. 49, 349-362; Han, Tetrahedron Asymmetry. 1994. 5, 2535-62).

[0032] Preferably the preparation of 2-deoxy-D-ribose (DRI) is carried out by oxidization of 2-deoxy-D-ribitol (DLR), e.g. with chromium oxide in pyridine.

[0033] In another embodiment of the invention, decarboxylation takes place by reacting (KDG) or its salts with an amino group-containing reagent Y-H leading to a compound of formula (V).



(V)

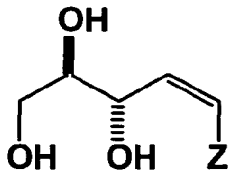
or its respective trans isomer or a protected form thereof, as a 2'-deoxynucleoside precursor. Y-H represents an amine with the hydrogen atom H bound to the nitrogen of the amino group.

[0034] In a preferred embodiment of the invention, the amino group-containing reagent represented by Y-H is a linear or cyclic secondary amine; a primary amine that possess a β-carbonyl group, preferably 3-amino-2-indolinone which was found to be effective for the decarboxylation of α-keto acids (Hanson, J. Chem. Education, 1987, 591-595). In each of these cases, -Y in formula (V) represents the respective nitrogen containing residue derived from these amino-group containing reagent.

[0035] Preferably, the compound of formula (V) represents an enamine produced via reaction of a linear or cyclic secondary amine as Y-H.

[0036] Preferred cyclic secondary amines are morpholine, pyrrolidine, piperidine, or N-methyl piperazine; preferred non-cyclic amines are those of the formula R_1-NH-R_2, wherein R_1 and R_2 independently represent a linear or branched alkyl group of 1-8, preferably 1 to 4 carbon atoms. Particularly preferred as a non-cyclic amine is diethylamine. Particularly preferred as a cyclic amine is morpholine.

[0037] The compound of formula (V) or its trans isomer or a protected form thereof can be further reacted with Z-H, wherein H represents a hydrogen atom and Z represents a leaving group, to produce a compound of formula (VI)



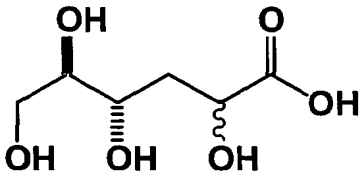
(VI)

or its respective trans isomer or a protected form thereof, as a 2'-deoxynucleoside precursor. Z-H is preferably water, in which case the compound of formula (VI) is DRI or a protected form thereof (keto-enol-tautomerism).

[0038] Preferably, the preparation of the compound of formula (V) is carried out by reacting KDG in benzene with the amine, e.g. morpholine under reflux using the method described in example 7, leading to 1-N-morpholino-3,4,5-tri-hydroxy-pentene-1. Acid catalysed hydrolysis with water yields 2-deoxy-D-ribose (DRI)

[0039] A general route to aldehydes via enamines from α—oxocarboxylic acids carrying β-hydrogens is described by Stamos (Tetrahedron Lett. 23 (1982), 459-462). Other methods for the preparation and hydrolysis of enamines have been described elsewhere (Stork, J. Am. Chem. Soc. 85 (1963), 207-222; Stamhuis, J. Org. Chem. 30 (1965), 2156-2160).

[0040] In another embodiment of the invention, KDG or its salt is converted to 3-deoxy-D-gluconic acid (DGN) and/or 3-deoxy-D-mannonic acid (DMN) represented by formula (VII) or the salts of these compounds

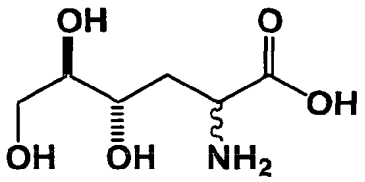


(VII)

[0041] The products resulting from this reaction undergo (oxidative) decarboxylation, preferably using hydrogen peroxide, to yield DRI. Production of a mixture of DGN and DMN or their salts takes place from KDG or its salts by reduction.

[0042] Preferably the preparation of 2-deoxy-D-ribose (DRI) is carried out by non-stereoselective reduction of 2-dehydro-3-deoxy-D-gluconic acid in water with sodium borohydride at room temperature using the method described for 2-keto-3-deoxyheptonic acid by Weissbach (J. Biol. Chem. 234 (1959), 705-709), followed by oxidative decarboxylation of 3-deoxy-D-gluconate and 3-deoxy-D-mannonate with hydrogen peroxide as described e.g. in US patent 3,312,683; Richards J. Chem. Soc. (1954), 3638-3640; Sowden J. Am. Chem. Soc. 76 (1954), 3541-3542.

[0043] In another embodiment of the invention, KDG or its salt is converted to 3-deoxy-D-glucosaminate (DGM) or 3-deoxy-D-mannosaminate (DMM) represented by formula (VIII) or the salts of these compounds



(VIII)

The products resulting from this reaction undergo (oxidative) decarboxylation, preferably using ninhydrin, to yield DRI. Production of a mixture of DGM and DMM or their salts takes place from KDG or its salts by reductive amination.

[0044] Preferably the preparation of 2-deoxy-D-ribose is carried out by non-stereoselective reductive amination of sodium or potassium 2-dehydro-3-deoxy-D-gluconate in aqueous solution with ammonia and sodium cyanoborohydride at room temperature, followed by oxidative decarboxylation of 3-deoxy-D-2-glucosaminate and 3-deoxy-D-2-mannosaminate with ninhydrin using the method described for the synthesis of 2-deoxy-D-allose by Shelton (J. Am. Chem. Soc. 118 (1996), 2117-2125; and Borch, J. Am. Chem. Soc. 93 (1971), 2897; Durrwachter, J. Am. Chem. Soc. 108 (1986), 7812 referenced therein).

[0045] Another aspect of the invention is a convenient and cost-effective method for preparing KDG either from D-gluconate (GCN) or from D-glucosaminate through the use of recombinant enzymes.

[0046] In a preferred embodiment of the method of the invention, the compound of formula (I) is produced in a preliminary step from a D-gluconate salt by the use of a D-gluconate dehydratase activity. Preferred salts are potassium or sodium D-gluconate. Preferably the D-gluconate dehydratase is encoded by a polynucleotide comprising the nucleotide sequence selected from the group consisting of:

(a) nucleotide sequences encoding a polypeptide comprising the amino acid sequence of SEQ ID N°2;
(b) nucleotide sequences comprising the coding sequence of SEQ ID N°1;
(c) nucleotide sequences encoding a fragment encoded by a nucleotide sequence of (a) or (b);
(d) nucleotide sequences hybridising with a nucleotide sequence of any one of (a) to (c); and
(e) nucleotide sequences which deviate from the nucleoside sequence of (d) as a result of degeneracy of the genetic code.

[0047] The enzymatic synthesis of KDG or its salts using D-gluconate dehydratase proceeds according to the following reaction: D-gluconate is converted into KDG by the elimination of one water molecule. The activity of a D-gluconate dehydratase has been characterized in different bacteria species e.g. in *Alcaligenes* (Kersters, Methods in Enzymology 42 (1975), 301-304); *Clostridium pasteurianum,* (Gottschalk, Methods in Enzymology 90 (1982), 283-287); *Thermoplasma acidophilum* (Budgen, FEBS Letters 196 (1986), 207-210) and *Sulfolobus solfataricus* (Nicolaus, Bio-

technology Letters 8(7) (1986), 497-500). The preferred D-gluconate dehydratase was identified by screening several collection strains for D-gluconate dehydratase activity. The gene encoding a D-gluconate dehydratase, which was designated gcnD was selected from a genomic library of *Agrobacterium tumefaciens* strain C58, and further inserted in a multi copy vector optimised for expression. It was shown that a crude extract from E. coli cells over-expressing the gcnD gene catalysed the total conversion of D-gluconate into KDG (see Example 2).

[0048] In a further preferred embodiment of the method of the invention, the compound of formula (I) is produced in a preliminary step from D-glucosaminate by the use of a D-glucosaminate deaminase activity. Preferably the D-glucosaminate deaminase is encoded by a polynucleotide comprising the nucleotide sequence selected from the group consisting of:

(f) nucleotide sequences encoding a polypeptide comprising the amino acid sequence of SEQ ID N°4;
(g) nucleotide sequences comprising the coding sequence of SEQ ID N°3;
(h) nucleotide sequences encoding a fragment encoded by a nucleotide sequence of (a) or (b);
(i) nucleotide sequences hybridising with a nucleotide sequence of any one of (a) to (c); and
(j) nucleotide sequences which deviate from the nucleoside sequence of (d) as a result of degeneracy of the genetic code.

[0049] The enzymatic synthesis of KDG or its salts using D-glucosaminate deaminase proceeds according to the following reaction: D-glucosaminate is converted into KDG by the elimination of one molecule water and one molecule of ammonia. The activity of a D-glucosaminate deaminase has been characterized in different bacteria species e.g. in *Pseudomonas fluorescens* (Iwamoto, Agric. Biol. Chem. 53 (1989), 2563-2569) *Agrobacterium radiobacter* (Iwamoto, FEBS Letters 104 (1979), 131-134; Iwamoto, J. Biochem. 91 (1982), 283-289), and its requirement for Mn^{2+} ion was shown (Iwamoto, Biosci. Biotech. Biochem. 59 (1995), 408-411).

[0050] The preferred D-glucosaminate deaminase was identified by screening several collection strains for D-glucosaminate deaminase activity. The gene encoding a D-glucosaminate deaminase, which was designated gmaA was isolated from *Agrobacterium tumefaciens* strain C58 by cloning a gene annotated as a putative D-serine deaminase. The gmaA gene was further inserted in a multi copy vector optimised for expression. It was shown that a crude extract from E. coli cells over-expressing the gmaA gene catalysed the conversion of D-glucosaminate into KDG (see Example 4).

[0051] These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices.

[0052] For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.google.de. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

Furthermore, the term "and/or" when occurring herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

EXAMPLES

Example 1. Cloning of a gene encoding a D-gluconate dehydratase from Agrobacterium tumefaciens strain C58 (CIP 104333)

[0053] *Agrobacterium tumefaciens* strain C58 (CIP 104333) was obtained from Institut Pasteur Collection (CIP, Paris, France). Chromosomal DNA was extracted and a D-gluconate dehydratase gene was amplified by PCR according to standard protocols using the following primers:

5'-CCCTTAATTAATGACGACATCTGATAATCTTC-3', depicted in SEQ ID N° 5;
5'-TTTGCGGCCGCTTAGTGGTTATCGCGCGGC-3', depicted in SEQ ID N° 6;
5'-CCCGGTACCATGACGACATCTGATAATCTTC-3',depicted in SEQ ID N° 7;

A first DNA fragment amplified using the two primers depicted in SEQ ID N° 5 and SEQ ID N° 6, was ligated into a pUC18-derived vector previously digested by PacI and NotI to yield the plasmid pVDM80. A second DNA fragment amplified using the two primers depicted in SEQ ID N° 6 and SEQ ID N° 7, was ligated into a pET29a vector (Novagen) previously digested by KpnI and NotI to yield the plasmid pVDM82. The nucleotide sequence of the cloned gene is depicted in SEQ ID N° 1 and the sequence of the polypeptide encoded by this gene is depicted in SEQ ID N° 2.

Example 2. Expression of a D-gluconate dehydratase activity in Escherichia coli and preparation of 2-dehydro-3-deoxy-D-gluconate from D-gluconate.

[0054] Competent cells of E. coli BL21 were transformed with the pVDM82 plasmid constructed as described in example 1 yielding strain +1289. Strain + 1289 was cultivated at 30°C in Luria-Bertani (LB) medium (Difco) containing 30 mg/l kanamycin until OD(600 nm) reached a value of 0.6. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a 0.5 mM final concentration. After a further cultivation period of 2 hours and 30 minutes, cells were collected by centrifugation and washed once with 20 mM sodium phosphate buffer pH 7.2. A cell extract was prepared by suspending about 5 g of cells in 10 ml of Tris-HCl 50 mM pH 8.5 buffer containing 10000 units lysozyme (Ready-Lyse, Epicentre, Madison, Wisconsin) and 1 mM EDTA, and incubating the suspension at 30°C for 15 minutes. Then 10000 kUnits deoxyribonuclease I (DNase I, Sigma) as well as 5 mM MgCl2 were added to the preparation which was incubated at 30°C for an additional period of 15 minutes. The cell extract thus obtained was kept frozen at -20°C before use. 1.5 ml of the cell extract was mixed with 2M sodium or potassium D-gluconate in a total volume of 10 ml. This preparation was incubated at 37°C after the pH has been adjusted to 8.5. The progression of 2-dehydro-3-deoxy-D-gluconate (KDG) synthesis was followed by analysing aliquots taken after increasing periods of incubation. Several dilution parts of these aliquots were deposited on silica plates and chromatographied in the following solvent system: isopropanol / water (90/10). A yellow spot of KDG (Rf ~0.40) was detected after revelation with p-anisaldehyde. KDG was also quantitated using a spectrophotometric assay based on the reaction with semicarbazide hydrochloride as described by Mac Gee (J. Biol. Chem. 1954. 210, 617-626). Typically, after a 30h period of incubation and using the spectrophotometric assay, KDG concentration ranged from 1.5 to 2 M.

The sodium or potassium 2-dehydro-3-deoxy-D-gluconate solution thus obtained could be used as such for further synthetic steps. 2-Dehydro-3-deoxy-D-gluconic acid could also be prepared from such a solution applying published protocols (Bender, Anal. Biochem. 1974. 61, 275-279). A crude preparation of a mixture of 2-dehydro-3-deoxy-D-gluconic acid and KCl could also be obtained by adding one equivalent of HCl to a potassium 2-dehydro-3-deoxy-D-gluconate solution which was then evaporated.

Example 3. Cloning of a gene encoding a D-glucosaminate deaminase from Agrobacterium tumefaciens strain C58 (CIP 104333)

[0055] *Agrobacterium tumefaciens* strain C58 (CIP 104333) was obtained from Institut Pasteur Collection (CIP, Paris, France). Chromosomal DNA was extracted and a D-glucosaminate deaminase gene was amplified by PCR according to standard protocols using the following primers:

5'-CCCTTAATTAATGCAGTCTTCTTCAGCTCTTC-3', depicted in SEQ ID N° 8;

5'-TTTGCGGCCGCCTAGTGAAAGAAGGTTGTGTAGAT-3', depicted in SEQ ID N° 9;

5'-AAATCATGACTATGCAGTCTTCTTCAGCTCTTCG-3', depicted in SEQ ID N° 10;

5'-TATAGATCTCTAGTGAAAGAAGGTTGTGTAGAT-3', depicted in SEQ ID N° 11;

A first DNA fragment amplified using the two primers depicted in SEQ ID N° 8 and SEQ ID N° 9, was ligated into a pUC18-derived vector previously digested by PacI and NotI to yield the plasmid pKDGb1. A second DNA fragment amplified using the two primers depicted in SEQ ID N° 10 and SEQ ID N° 11, was ligated into a pQE60 vector (Qiagen) previously digested by BspH1 and BglII to yield the plasmid pEP18. The nucleotide sequence of the cloned gene is depicted in SEQ ID N° 3 and the sequence of the polypeptide encoded by this gene is depicted in SEQ ID N° 4.

Example 4. Expression of a D-glucosaminate deaminase activity in Escherichia coli and preparation of 2-dehydro-3-deoxy-D-gluconic acid from D-glucosaminate

[0056] Competent cells of E. coli MG1655 were transformed with the pEP18 plasmid constructed as described in example 1 and pREP4 (Qiagen) yielding strain +1068. Strain + 1068 was cultivated at 37°C in LB medium containing 30 mg/l kanamycin and 100 mg/l ampicillin until OD(600 nm) reached a value of 0.6. Then IPTG was added to a 0.5 mM final concentration. After a further cultivation period of 2 hours and 30 minutes, cells were collected by centrifugation and washed once with 20 mM sodium phosphate buffer pH 7.2. A cell extract was prepared using the protocol described in example 2.

[0057] 2 ml of the cell extract was mixed with 100 mM sodium or potassium D-glucosaminate and 0.1 mM pyridoxal phosphate in a total volume of 5 ml. This preparation was incubated at 37°C after the pH has been adjusted to 7.5. The progression of 2-dehydro-3-deoxy-D-gluconate (KDG) synthesis was followed using the protocols described in example 2. Typically, after a 30h period of incubation and using the spectrophotometric assay described in example 2, KDG concentration ranged from 50 to 100 mM.

Example 5. Preparation of 2-deoxy-D-ribonate from 2-dehydro-3-deoxy-D-gluconate

[0058] 0.5 ml of a 31% hydrogen peroxyde solution were added to 5 ml of a 1M potassium 2-dehydro-3-deoxy-D-gluconate (KDG) solution at 25°C. The progression of KDG decarboxylation was followed both by the observation of bubbles resulting from the release of carbon dioxide and by the disappearance of KDG using the thin layer chromatography protocol described in example 2. Typically, after a 3h period of reaction the concentration of residual KDG was less than 10 mM.

Example 6. Preparation of 2-deoxy-D-ribitol from 2-deoxy-D-ribonolactone

[0059] 0.2 g of Rhodium (5 % on carbon) catalyst was added to an aqueous solution of 1 g 2-deoxy-D-ribonolactone prepared following a method described by Deriaz (J. Chem. Soc. (1949), 1879-1883) for the synthesis of 2-deoxy-L-ribonolactone. Hydrogenation of 2-deoxy-D-ribonolactone was performed at 130°C under a pressure of 80 bars. The solution obtained after filtration of the reaction mixture was evaporated. The residue was dissolved in ethyl acetate and further purified by chromatography on a silica column. The solvent was removed in vacuo leading to a yellow oil (yield 85%).The compound thus obtained was identical with 2-deoxy-D-ribitol obtained by reduction of 2-deoxy-D-ribose as described by Rabow (J. Am. Chem. Soc. 122 (1999), 3196-3203).

Example 7. Preparation of 1-N-morpholino-3,4,5-trihydroxypentene-1 from 2-dehydro-3-deoxy-D-gluconate.

[0060] 2 g of 2-dehydro-3-deoxy-D-gluconic acid were suspended in 150 ml benzene. 1.1 ml morpholine and 100 mg p-toluenesulfonic acid were added to the suspension and the reaction mixture was refluxed for 3 hours. Water formed by this reaction was removed by distillation. Benzene was decanted. Solid compounds attached to the vessel were collected, washed with acetone and dried. The main compound present in this preparation (yield 40%) was further purified by column chromatography on a silica column using a gradient of methanol in chloroform. Fractions containing 1-N-morpholino-3,4,5-trihydroxypentene-1 were pooled and solvent was removed in vacuo.
^{1}H-NMR (D_20): δ = 3.15 ppm (4H, t, morpholine), 3.8 ppm (4H, t, morpholine), 3.4 to 4 ppm, (4H, m, 5a-H, 5b-H, 4-H, 3-H), 6.3 and 6.8 ppm (2H, 2d, 1-H and 2-H, J = 4 Hz).

SEQUENCE LISTING

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<120> PRODUCTION OF 2'-DEOXYNUCLEOSIDES AND 2'-DEOXYNUCLEOSIDE PRECURSORS FROM 2-DEHYDRO-3-DEOXY-D-GLUCONATE

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        115                 120                 125

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Gly Gly Leu Thr Ser Ala Arg Arg Leu Arg Met Leu Ala Asp Ala Phe
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Phe Ile Glu Val Gly Val Gly Arg Thr Gly Ala Arg Thr Leu Asn Val
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Val Cys Glu Ala Asp Gly Asn Ala Thr Leu Leu Leu Arg Ser Gly Ala
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Asp Gln His Ala Phe Leu Ser Phe Gly Asn Gly Ser Ser Leu Ala Ile
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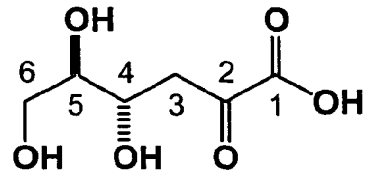
Claims

1. A method for producing 2'-deoxynucleosides or 2'-deoxynucleoside precursors from a compound of formula (I) or its salts



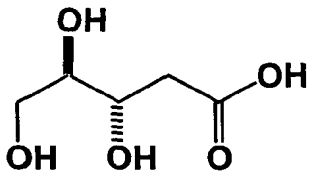
(I)

or a protected form thereof in a process comprising a decarboxylation step.

2. The method of claim 1 wherein the decarboxylation step cleaves the C1-C2 bond of the compound of formula (I) or its salts or a protected form thereof.

3. The method of claim 1 or 2, wherein the decarboxylation step is directly carried out on the compound of formula (I) or its salts or a protected form thereof.

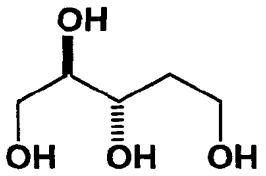
4. The method of any of claims 1 to 3, wherein the decarboxylation step takes place by reacting the compound of formula (I) or its salts or a protected form thereof with hydrogen peroxide to yield a compound of formula (II) or its salts



(II)

or a protected form thereof as a 2'-deoxynucleoside precursor.

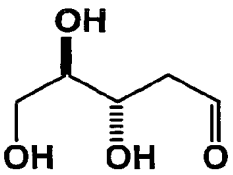
5. The method of claim 4, further comprising the conversion of the compound of formula (II) or its salts or a protected form thereof into a compound of formula (IV)



(IV)

or a protected form thereof as a 2'-deoxynucleoside precursor.

6. The method of claim 4, further comprising the conversion of the compound of formula (II) or its salts or a protected form thereof into a compound of formula (III)

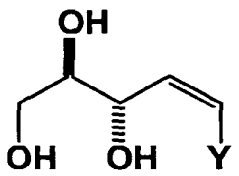


(III)

or a protected form thereof as a 2'-deoxynucleoside precursor.

7. The method of claim 6, comprising the conversion of the compound of formula (II) or its salts or a protected form thereof into the compound of formula (IV) or a protected form thereof as an intermediate which is then converted to the compound of formula (III) or a protected form thereof.

8. The method of any of claims 1 to 3, wherein the decarboxylation step takes place by reacting the compound of formula (I) or its salts or a protected form thereof with an amine Y-H, wherein H represents a hydrogen atom bound to the nitrogen atom of the amino group, to produce a compound of formula (V),



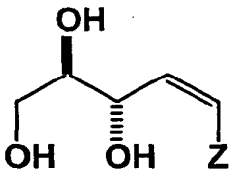
(V)

or its respective trans isomer or a protected form thereof, as a 2'-deoxynucleoside precursor.

9. The method of claim 8, wherein Y-H represents a linear or cyclic secondary amine.

10. The method of claims 8 or 9, wherein Y-H is morpholine, pyrrolidine, piperidine, N-methyl piperazine or diethylamine.

11. The method of any of claims 8 to 10, further comprising the step of reacting a compound of formula (V) or its trans isomer or a protected form thereof with Z-H, wherein H represents a hydrogen atom and Z represents a leaving group, to produce a compound of formula (VI)

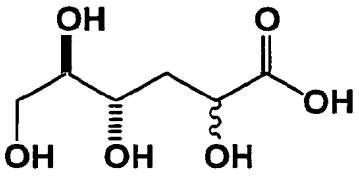


(VI)

or its respective trans isomer or a protected form thereof, as a 2'-deoxynucleoside precursor.

12. The method of claim 11, wherein Z-H is water, to produce a compound of formula (III) or a protected form thereof as a 2'-deoxynucleoside precursor.

13. The method of claim 1 or 2, wherein the compound of formula (I) or its salts or a protected form thereof is converted to a compound of formula (VII), or its salts or a protected form thereof or a mixture of the respective epimers,



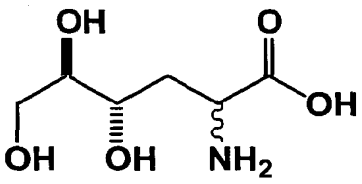
(VII)

which is then decarboxylated to yield a compound of formula (III) or a protected form thereof as a 2'-deoxynucleoside precursor.

14. The method of claim 13, wherein the conversion of (I) or its salts or a protected form thereof to (VII) or a protected form thereof takes place by reduction with sodium borohydride.

15. The method of claim 13 to 14, wherein the decarboxylation step takes place by reaction with hydrogen peroxide.

16. The method of claim 1 or 2, wherein the compound of formula (I) or its salts or a protected form thereof is converted to a compound of formula (VIII), or its salts or a protected form thereof or a mixture of the respective epimers,



(VIII)

which is then decarboxylated to yield a compound of formula (III) or a protected form thereof as a 2'-deoxynucleoside precursor.

17. The method of claim 16, wherein a compound of formula (VIII) or a protected form thereof or a mixture of the respective epimers is reacted with ninhydrin, thereby leading to the compound (III) or a protected form thereof.

18. The method of claim 16 or 17, wherein the conversion of (I) or its salts or a protected form thereof to (VIII) or a protected form thereof takes place by reductive amination with ammonia and sodium cyanoborohydride.

19. The method of any of claims 1 to 18, wherein the protective group(s) are independently chosen from acetate ester, benzoate ester, allyl ether, benzyl ether, trityl ether, ter-butyldimethylsilyl (TBDMS) ether, isopropylidene or a benzylidene acetal.

20. The method of any one of claims 1 to 19, comprising the preliminary step of producing the compound of formula (I) from a D-gluconate salt by the use of a gluconate dehydratase activity.

21. The method of claim 20, wherein the D-gluconate salt is potassium or sodium D-gluconate.

22. The method of claims 20 or 21, wherein the gluconate dehydratase is encoded by a polynucleotide comprising the nucleotide sequence selected from the group consisting of:

(a) nucleotide sequences encoding a polypeptide comprising the amino acid sequence of SEQ ID N°2;
(b) nucleotide sequences comprising the coding sequence of SEQ ID N°1;
(c) nucleotide sequences encoding a fragment encoded by a nucleotide sequence of (a) or (b);
(d) nucleotide sequences hybridising with a nucleotide sequence of any one of (a) to (c); and
(e) nucleotide sequences which deviate from the nucleoside sequence of (d) as a result of degeneracy of the genetic code.

23. The method of any one of claims 1 to 19, comprising the preliminary step of producing the compound of formula (I) from D-glucosaminate by the use of a glucosaminate deaminase activity.

24. The method of claim 23, wherein the glucosaminate deaminase is encoded by a polynucleotide comprising the nucleotide sequence selected from the group consisting of:

(a) nucleotide sequences encoding a polypeptide comprising the amino acid sequence of SEQ ID N°4;
(b) nucleotide sequences comprising the coding sequence of SEQ ID N°3;
(c) nucleotide sequences encoding a fragment encoded by a nucleotide sequence of (a) or (b);
(d) nucleotide sequences hybridising with a nucleotide sequence of any one of (a) to (c); and
(e) nucleotide sequences which deviate from the nucleoside sequence of (d) as a result of degeneracy of the genetic code.

25. Use of a polynucleotide as defined in claim 22 or of a gluconate dehydratase encoded by such a polynucleotide in a method according to claims 20 or 21.

26. Use of a polynucleotide as defined in claim 24 or of a glucosaminate deaminase encoded by such a polynucleotide in a method according to claim 23.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 03 01 3457

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	SHELTON M C ET AL: "2-Keto-3-deoxy-6-phosphogluconate aldolases as catalysts for stereocontrolled carbon-carbon bond formation" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 118, no. 9, 6 March 1996 (1996-03-06), pages 2117-2125, XP002263455 ISSN: 0002-7863 Scheme 6 ---	1	C07H7/027 C07H1/00
A	US 5 872 247 A (DUFLOT PIERRICK ET AL) 16 February 1999 (1999-02-16) exemples ---	1	
A	US 5 846 794 A (DELOBEAU DIDIER ET AL) 8 December 1998 (1998-12-08) exemple 3 ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	H. KILIANI, H. NAEGELL: "Ueber Meta- und Parasaccharin" CHEM. BER., vol. 35, 1902, pages 3528-3533, XP002267849 * page 3531 - page 3532 * -----	13	C07H

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	23 January 2004	de Nooy, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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23-01-2004

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